Standard and biochemical toxicological effects of zinc pyrithione in *Daphnia magna* and *Daphnia longispina*

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Graphical abstract
Highlights

- Exposure to ZnPT resulted in cholinesterasic inhibition in both Daphnia species
- Exposure to ZnPT may trigger oxidative and neurotoxic effects in both Daphnia species
- ZnPT is potentially more toxic to D. magna than to D. longispina
- Zinc pyrithione may constitute a potential threat to aquatic organisms, namely crustaceans

Abstract

Chemical toxicity in the environment may be the consequence of exposure of living organisms to multiple substances, with distinct putative effects. Among this multiplicity of chemicals that occur in the wild, pharmaceutical drugs and antifoulers are prone to exert toxic effects on non-target organisms. To characterize the toxicity elicited by a compound of this specific class, the present study used standard and biochemical-based tools to quantify the toxic response of the antifouler and antidandruff zinc pyrithione in Daphnia magna and Daphnia longispina. The analysed parameters were immobility, reproduction, behavioral alteration (swimming patterns), anti-oxidant defense (catalase activity), metabolism (GSTs activities), and neurotoxicity (ChE activity) after exposure to sublethal concentrations of this drug. Exposure to zinc pyrithione has been shown to have neurotoxic and oxidative effects, with changes in swimming behavior. There were no changes in reproductive traits of exposed individuals, from both species. The obtained data demonstrate that ecologically relevant levels of zinc pyrithione can deleteriously alter critical parameters in two distinct freshwater microcrustacean species, although with distinct toxicity patterns and outcomes.
Keywords: Drugs, crustaceans, biomarkers, population effects, antifouling compounds, sublethal effect

Introduction

Continued use of pharmaceuticals drugs results in persistent loading of aquatic ecosystems with parental compounds and their metabolites (Beek et al., 2016; Pugajeva et al., 2017). Pharmaceutical residues can reach the environment through different routes, being the most common through effluents from wastewater treatment plants (WWTPs), since after being consumed, the drugs are excreted, in their original form or metabolized, by faeces and urine. The environmental concentration levels of pharmaceutical residues vary usually from ng/l to µg/L (Pugajeva et al., 2017) and are related to their consumption pattern, their persistence in the environment, the removal efficiency in WWTPs, and seasonality (Carvalho et al., 2009). These concentrations may cause damage to various levels of biological organization (cell, organ, individual, populations and ecosystems) (Bila & Dezotti, 2003; Gaffney et al., 2014) and effects such as changes in gene expression (genotoxicity) and immunological changes (immunotoxicity), endocrine system disorders (reduced embryonic production, in sperm motility, male feminization and masculinization of females), behavioral modification (in swimming performance and predator escape behavior, light/dark preference) are reported in the literature (Salibián, 2013; Gaffney et al., 2014; Marin-Morales et al., 2016). In addition, a large body of evidence demonstrates the occurrence of biochemical disorders such as oxidative stress, lipid peroxidation, and neurotoxicity, also induced by pharmaceutical drugs (Nunes et al., 2006; Antunes et al., 2013; Jasinska et al., 2015; Oliveira et al., 2015; Mezzelani et al., 2016). These effects arise from the fact that the drugs have biological activity and are active and persistent, that is, they are synthesized and produced to alter biological functions and maintain their chemical properties for the time necessary to assure the exercise of the therapeutic purpose (Nunes, 2011).

Zinc pyrithione (1-hydroxypyridine-2-thione, zinc salt) is an organometallic biocide with bactericidal, fungicidal and algicidal activity. It is used as an active ingredient in medical and hygienic products (such as anti-dandruff shampoos and soaps; Schwartz et al., 2011) and in antifouling paints to prevent the formation of biofilms on surfaces in contact with
the aquatic environment (Turley et al., 2009; Ohji & Harino, 2017), including in vessels/boats or aquatic and submerged structures. Antifouling biocides are among the most important compounds present in the environment as they are used to prevent the spontaneous colonization of organisms in structures immersed in water, such as ship hooves, buoys, oil rigs, aquaculture equipment, among others (Almeida et al., 2007; Konstantinou & Albanis, 2004; Soroldoni et al., 2017). Zinc pyrithione can enter the environment through wastewater treatment plants or during ship repair, cleaning or painting processes in shipyards and docks. Painting of submerged structures may result in the release of particles from antifouling paints that can be transported dissolved in water and deposited in sediments (Soroldoni et al., 2017). Sediments containing particles of antifouling paints may undergo resuspension and leach these compounds, making them available to aquatic organisms (Jung et al., 2017). Data on the environmental fate of zinc pyrithione are scarce, and may be associated with its chemical instability; zinc pyrithione reacts with other metal ions (namely copper) by translocation, and is easily transformed into copper pyrithione, giving rise to more toxic and stable forms (Madsen et al., 2000) in addition, there are a number of analytical difficulties for its quantification (Thomas & Brooks, 2010). However, zinc pyrithione levels of 15.9 μg/l have already been detected in the Mersey estuary, UK (Mackie et al., 2004), showing that environmental effects should be considered since some previous studies point to its potential for being bioaccumulated by some organisms (Marcheselli et al., 2010), and for its toxicity. Studies to evaluate the acute toxicity of zinc pyrithione in aquatic organisms have resulted in EC₅₀ values indicating high toxicity to aquatic organisms such as Nitzschia pungens (EC₅₀ = 5.5 μg/l), Artemia sp. (LC₅₀ = 3.2 mg/l) (Jung et al., 2017). The acute (48h) assay performed with the fish Oryzias latipes exposed to low concentrations of zinc pyrithione (1-10μg/l) resulted in morphological and respiratory abnormalities (Ohji & Harino, 2017). Among the main reported effects of zinc pyrithione is embryotoxicity even at extremely low levels, alteration in the expression of heat shock proteins, genotoxicity and neurotoxicity (Nunes et al., 2015). The photodegradation of ZnPT (and not ZnPT itself) may result in the formation of two chemicals, namely 2,2′-dipyridyldisulfide [(PS)₂] and 2,2′-dithiobispyridine-N-oxide [(PT)₂], known for their AChE-inhibiting activity (Mochida et al., 2009).
Several species of *Daphnia* have been used as organisms in toxicity tests (Forrón et al., 2008). *Daphnia longispina* (O.F. Müller, 1776), commonly found in Portuguese freshwater (reservoirs and lacustrine systems), has been used as an indicator species to evaluate water quality (Neves et al., 2015). *Daphnia magna* (Straus) is used as a model organism in several acute and chronic toxicity testing protocols of international environmental protection organizations (OECD, ASTM, US EPA) to evaluate parameters such as mortality, reproduction, and changes in behavior (Persoone et al., 2009). The species *Daphnia magna* and *Daphnia longispina* have been used as model organisms in drug trials (Dave & Herger, 2012; Du et al., 2016), metals (Biesinger & Christensen, 1972; Martins et al., 2017), herbicides (Neves et al., 2015) and others.

Behavioral and biochemical data on the effects of zinc pyrithionate are scarce or non-existent. The present study aimed to evaluate the effects of zinc pyrithione, through standardized tests, using parameters such as mortality, reproduction, swimming behavior and determination of changes induced at the biochemical level by means of biomarkers. The effects were evaluated in two distinct species of *Daphnia* (namely *D. magna* and *D. longispina*), to increase the robustness of the set of data with a more comprehensive number of analytical biomarker tools in the evaluation of toxicity, allowing also to evaluate potential interspecific differences.

**MATERIALS AND METHODS**

**Culture of test organisms**

Animals genotypically well identified, from healthy cultures, kept under appropriate laboratory conditions (light, temperature, medium, food, number of animals per culture and unit volume, according to the procedures and recommendations of OECD guidelines (OECD, 2012), were used in the present study. *Daphnia magna* (clone K6) and *Daphnia longispina* (clone E-89) were obtained from monoclonal cultures of the Applied Ecology and Ecotoxicology Laboratory – Applie, of the Center for Environmental and Marine Studies (CESAM), University of Aveiro. For the beginning and renewal of the culture, animals from the 3rd to the 5th broods were used. The animals were cultured in synthetic hard water medium (ASTM, 2007; USEPA, 1993), exposed to a photoperiod of 16h-light:8h-dark, with a temperature of 21°C (± 2°C), without aeration (OECD, 2012). The medium was changed three times a week, with the addition of seaweed extract.
Ascophyllum nodosum (Antunes et al., 2003; Loureiro et al., 2011) and Raphidocelis subcapitata algae, grown under controlled conditions (Stein, 1973). Cultures were fed with these algal species, in an amount up to a $3.0 \times 10^5$ cells/ml/Daphnia magna and $1.5 \times 10^5$ cells/ml/Daphnia longispina (Antunes et al., 2003; Loureiro et al., 2011).

**Chemical compounds and test concentrations**

Zinc pyrithione ($C_{10}H_8N_2O_2S_2Zn$; CAS: 13463-41-7) with purity of 95% were purchased from Sigma-Aldrich. Stock solutions of 4mg/l were prepared by dilution in ultrapure water (MILLI-Q®) for each drug prior to each assay. The test solutions were prepared from the stock solution by dilution in ASTM medium.

**Mortality determinations**

Mortality was evaluated considering the number of immobile neonates for 15 seconds under slight agitation, after 48 hours of exposure to different concentrations of the analyzed compounds (OECD, 2004). Acute toxicity tests were performed with both species according to the protocol of the Organization for Economic Cooperation and Development - OECD, Guideline 202 (OECD, 2004). Neonates of D. magna and D. longispina up to 24 hours of age were exposed to the following concentrations of zinc pyrithione ($\mu$g/l): 0 (control), 82, 102.5; 128.2; 160.2; 200.2; 250.3; 312.8, and 391 $\mu$g/l, with five replicates, containing 50 ml of each concentration and five neonates per replicate. These concentrations used for the mortality determinations were selected according to the results obtained for previously performed range finding tests (data not shown). The assays were conducted under the same conditions described for cultures, without the addition of food. The mortalities of the individuals were recorded at 24h and 48h for statistical analysis and determination of $EC_{50}$ values.

**Exposure of organisms for the determination of biomarkers**

For the evaluation of the putative toxic effects in terms of biochemical parameters, an acute exposure (48h) based on the OECD-Guideline 202 (OECD, 2004), adapted to
sublethal concentrations, was performed. Neonates (less than 24 hours old and born between the 3rd and 5th broods) of *D. magna* and *D. longispina* were exposed to several zinc pyrithione concentrations (μg/l): 0 (control); 5; 7; 9.8; 13.7, and 19.8 μg/l. These concentrations were selected considering the environmental concentrations found in the literature (Thomas et al., 2001; Mackie et al., 2004).

Different exposures were made for the determination of each biochemical marker. For *D. magna*, 50 neonates were exposed in vials containing 170 ml of the test solutions, with five replicates per concentration. For *D. longispina* the exposure was conducted with 100 animals per replicate. At the end of each exposure, the neonates were collected in Eppendorf microtubes and stored at -80°C for further processing.

Sample processing

For enzymatic determinations of catalase, the samples were thawed on ice, and a volume of 1ml of 50mM phosphate buffer, pH = 7 with 0.1% Triton X-100 was added to each sample. Samples were homogenized in an ultrasound homogenizer and centrifuged at 15000g for 10 minutes at 4°C. The supernatants after centrifugation were collected in properly identified Eppendorf microtubes and stored at -80°C.

Quantification of catalase (CAT) activity

Catalase is an enzyme responsible for the decomposition of H$_2$O$_2$ in H$_2$O + O$_2$ and peroxidic activity, where H$_2$O$_2$ consumption is due to the oxidation of hydrogen donors (methanol, formic acid and phenols). The method consists in the monitoring of this decomposition, determined spectrophotometrically at 240 nm ($\varepsilon_{240} = 0.00394 \pm 0.0002$ liters mmol$^{-1}$mm$^{-1}$), in which the decrease in absorbance ($\Delta$ 240) (Aebi, 1984) is observed. The enzymatic activity was expressed in nmoles of H$_2$O$_2$ consumed per minute per milligram of protein.

Quantification of the activity of the isoenzymes glutathione S-transferases (GSTs)

GSTs were determined after thawing the samples by adding 1ml of 50mM phosphate buffer, pH = 7 with 0.1% Triton X-100 for homogenization. Samples were then centrifuged
at 15.000g for 10 minutes at 4°C. The supernatants after centrifugation were collected in Eppendorf microtubes properly identified. The activity of glutathione S-transferases (GSTs) was determined according to (Habig et al., 1974). GSTs catalyze the conjugation of glutathione in its reduced form (GSH) with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), forming a thioether (ε = 9.6 mM⁻¹cm⁻¹) whose formation is observed by increase of the absorbance detected spectrophotometrically at 340 nm. Enzymatic activities were expressed in nmol of thioether produced per minute per milligram of protein (Habig et al., 1974).

Quantification of the activity of the enzyme cholinesterases (ChEs)

For the determination of cholinesterase activity, the samples were homogenized with 0.1M phosphate buffer, pH = 7.2 and centrifuged at 3330g for 3 minutes. After centrifugation, the supernatants were collected in properly identified Eppendorf microtubes, and stored at -80°C. The quantification of the ChEs enzymatic activity was performed by the Ellman method (Ellman et al., 1961) adapted to 96-well microplates (Guilhermino et al., 1996). The enzymes cholinesterases are responsible for the degradation to acetylthiocholine in acetate + thiocoline. In this method, the activity of the enzyme is measured by recording the increase in absorbance at 414 nm over time, which occurs with the increase in the yellow color produced when thiocoline is complexed with DTNB (dithiobisnitrobenzoate). The enzymatic activity was expressed as nmol of the complex formed per minute per milligrams of protein.

Determination of total soluble protein concentration

The protein concentration of samples was determined by the Bradford method adapted to 96-well microplates using standard 1 mg/ml γ-globulin (Bradford, 1976; Qi et al., 2017). This method is based on the binding of a dye (Bradford's reagent) to soluble proteins, giving rise to a stable complex, whose presence is detectable at 595 nm. This quantification allows expressing the enzymatic activity as a function of the protein content of the sample.
**Behavioral evaluation**

Acute exposures (48h) to sublethal concentrations of zinc pyrithione were performed to evaluate the behavior of neonates (age <24h) and adults (age 6 days) of *D. magna* and *D. longispina* (ASTM, 2007; OECD, 2004). The animals were exposed to the following concentrations of zinc pyrithione (μg/l): 0 (control); 5; 7; 9.8; 13.7, and 19.8 μg/l, selected considering their ecological relevance as a function of the environmental concentrations already mentioned in the literature, around 15 μg/l (Thomas et al., 2001; Mackie et al., 2004). Six well microplates with approximately 10 ml of test solution per well were used for each concentration of test substance, and one organism was randomly added to each well. Each plate was composed of one control and one concentration to be tested, totaling 20 organisms per concentration. The assays were maintained under the same culture conditions, without food. After exposure, the organisms were transferred to 24-well plates with 2 ml of the respective test solutions and acclimated for 10 minutes prior to the analysis of their behavior by the monitoring system. Each plate was composed of four control organisms and 20 organisms of one of the concentrations tested. Being light sensitive organisms, individuals of species from the *Daphnia* genus are likely to react to light stimuli (Ringelberg, 1964; van Gool; 1997; Effertz and von Elert, 2014). After acclimatization, the animals were subjected to alternating cycles of light and darkness (first light cycle – first dark cycle – second light cycle – second dark cycle; each cycle had a duration of 300 seconds), totaling 20 minutes (1200 seconds). The measured endpoints were average swimming time (ST), and average swimming distance (SD). This experimental analysis was based on the studies conducted by Alkimin et al. (2020), and Dionísio et al. (2020), who validated this analytical time frame, and the measurement of such behavioral traits. Changes in swimming behavior can be very subtle, difficult to observe with the naked eye, and digital analysis and video recording appear as sensitive tools capable of monitoring various parameters related to mobility (Dodson et al., 1995); in our case the evaluation of the locomotor behavior was performed by using an automated video recording system (Viewpoint Zebrabox®), equipped with internal LED (recording light) and infrared (dark recording) lighting, with a camera mounted for the detection of movements.
Reproduction assay

To assess the effects of zinc pyrithione on the reproductive performance of *D. magna* and *D. longispina*, neonates (less than 24 hours) were chronically exposed to sublethal concentrations of zinc pyrithione (μg/l): 0 (control); 5; 7; 9.8; 13.7, and 19.8μg/l. The tests were carried out according to the guideline OECD 211 (OECD, 2012), for a period of 21 days, with semi-static flow, photoperiod of 16h-light: 8h-dark, with temperature of 21°C (± 2°C), with addition of food under the same culture conditions (Antunes et al., 2003; Loureiro et al., 2011). To carry out the tests, 10 vials per concentration (containing 50 ml of the test solution) were used, and one organism per vial (OECD, 2012). The total number of offspring at the end of the test was recorded for statistical analysis.

Statistical analysis

For EC\textsubscript{50} calculation, the results were analyzed by probit analysis to the EC\textsubscript{50} for immobility, with 95% confidence limits (p=0.95) (OECD, 2004). The analysis was performed using the IBM SPSS Statistics software (version 25). The obtained data were previously analyzed to guarantee uniformity of variance and normality (Shapiro-Wilk). The biochemical and behavioral parameters (swimming and reproduction) were analyzed by analysis of variance (One-Way Anova), followed, when necessary by a Dunnett test to discriminate significant differences of the exposed groups concentrations of the drugs in relation to the control treatment. The level of significance was 0.05. The data are presented as mean and respective standard error. The analysis was performed using the software Sigmaplot (version 12.5) and IBM SPSS Statistics (version 25).

RESULTS

Mortality

Acute toxicity was assessed (immobility) by calculating EC\textsubscript{50} values with the respective confidence intervals (Table 1). For *D. magna* the calculated values were EC\textsubscript{50}=155,9μg/l and for *D. longispina* EC\textsubscript{50}=256,4μg/l.
Biomarkers

Catalase (CAT)

Zinc pyrithione significantly increased the activity of the catalase enzyme (Figure 1) in individuals of *D. magna* exposed to concentrations of 9.8 and 19.2 μg/l, (F=11.55; p<0.001; d.f.=5;24). There were no significant differences in catalase activity in organisms of *D. longispina* after exposure to zinc pyrithione (F=1.25; p=0.317; d.f.=5; 24).

Glutathione S-transferases (GSTs)

Zinc pyrithione (Figure 2) caused a significant increase in GSTs activity in *D. magna* at the highest tested concentrations (13.7 and 19.2 μg/l) (F=32.70, p=0.05; d.f.=5;24). In *D. longispina* an inhibition of activity was observed in organisms exposed to the highest concentration (19.2 μg/l) of zinc pyrithione (F=1.87; p<0.05; d.f.=5; 24).

Cholinesterases (ChEs)

Significant inhibition of cholinesterases was observed in *D. magna* after being exposed to zinc pyrithione (Figure 3) (F=6.60; p<0.05; d.f.=5;24) at all concentrations tested, and in *D. longispina* the same trend occurred but only for organisms exposed to the concentration of 7μg/l, (F=2.20, p<0.05, d.f.=5;24).

Swimming behavior

Behavioral changes were observed for the two species studied after being exposed to ZnPT (Figures 4 and 5). Results of the statistical analysis are summarized in Table 2, and the comparative results between concentrations and control (Dunnett’s test) are depicted in Table 3.

There were no significant differences between animals exposed to zinc pyrithione and control neonates of *D. magna*. In adults, a decrease in average swimming time (ST) was observed at concentrations 5; 9.8; 13.7; 19.2 and 13.7μg/l, for the light cycles, and for animals exposed to the concentrations of 9.8 and 19.2μg/l, for the dark cycles. A decrease in average swimming distance (SD) was observed for individuals exposed to concentrations 5; 13.7 and 19.2 μg/l for light cycles, and at concentrations of 9.8 and 19.2
µg/l for dark cycles. In neonates of D. longispina, exposures to concentrations 5; 9.8 and 13.7µg/l of zinc pyrithione, resulted in a decrease in ST for light cycles. No significant change was observed for animals during the dark cycles. Decreased SD was observed in animals exposed to a concentration of 5µg/l for the first light cycle, and at concentrations 5; 9.8 and 13.7µg/l for the dark cycles. In adults, concentrations of 5 and 7µg/l resulted in significant differences, with a decrease in ST for light cycles and a concentration of 5µg/l for the first dark cycle. SD was changed at the lowest concentration (5µg/l), for the first clear cycle.

Reproduction

The data obtained in the reproductive tests of D. magna (p = 0.419) and D. longispina (p = 0.003) exposed to zinc pyrithione did not result in statistically significant differences in relation to the control (Figure 6).

DISCUSSION

Acute toxicity (immobility) of zinc pyrithione has already been evaluated by Bao et al., (2011), for aquatic species: cyanobacteria - Chroococcus minor (7 days, EC₅₀ = 51µg/l), Synechococcus sp. (96h, EC₅₀ = 22µg/l); microalgae -Skeletonema costatum (96h, EC₅₀ = 1.7µg/l), Thalassiosira pseudonana (96h, EC₅₀ = 0.5µg/l), Pyrocystis lúnula (24h, EC₅₀ = 44µg/l); sea anemone - Aiptasia sp. (96h, LC₅₀ = 410µg/l); polychaetes -Hydroides elegans - larvae (48h, LC₅₀ = 7.6µg/l); juvenile (96h, LC₅₀ = 29µg/l), Tigriopus japonicus - adult (96h, LC₅₀ = 170µg/l) and fish -Oryzias melastigma- larvae (96h, LC₅₀ = 8.2µg/l) (Bao et al., 2011). The here-calculated EC₅₀ values for D. magna (155.9µg/l) and D. longispina (256.4µg/l) were of the same order of magnitude (in the range of the µg/l) of the values obtained for zooplanktonic crustaceans, namely Balanus amphitrite, Elasmopus rapax, Tigriopus japonicus, reported by Bao et al. (2011). EC₅₀ values in the order of the µg/l, observed in the literature, and confirmed in this work, indicate that zinc pyrithione is toxic to aquatic organisms, being this parameter a crucial comparative tool for the evaluation of the ecological impact of this compound.
Data about environmental concentrations of zinc pyrithione are scarce or non-existent. However, pyrithione was detected in samples obtained from marine waters in the UK at concentrations of 15.9 μg/l (Mackie et al., 2004), indicating that possible environmental levels can cause toxic effects in sensitive organisms, such as microalgae and polychaetes, whose EC50 range from 0.5 to 1.7μg/l (Bao et al., 2011). Experiments carried out with mussels of the species *Mytilus galloprovincialis* collected in the port area of Italy and exposed to sublethal doses of zinc pyrithione for 7 days demonstrated the occurrence and persistence of this compound in the wild. Control animals showed considerable baseline levels of zinc pyrithione, indicating that it is naturally occurring at the sampling site, in amounts high enough to be absorbed and detected in individuals collected from the wild population. In the exposed animals, the accumulation of the compound was proportional to the exposure concentration and time, indicating its potential threat to the organisms of the coastal environment (Marcheselli et al., 2010).

The mechanisms of toxicity of zinc pyrithione are not yet clearly elucidated. Studies point to the inhibition of ATP synthesis in prokaryotic cells and membrane depolarization (Dinning et al., 1998). However, there are no data in the literature that demonstrate that these mechanisms could be responsible for the acute effects of zinc pyrithione. Another factor to be considered in the interpretation of the acute toxicity of zinc pyrithione is related to the possibility of formation of toxic metabolites by light. Zinc pyrithione (and one of its potential derivatives, copper pyrithione) is rapidly transformed by photolysis under direct sunlight; however in conditions where light is limited, these compounds may accumulate in sediments (Du et al., 2016). Despite this possibility, Sánchez-Bayo & Goka (2006) compared the acute effects of zinc pyrithione in the crustaceans *Cypretta seuratti*, *Ilyocypris dentifera*, *Chydorus sphaericus* and *Daphnia magna*, in exposures performed in the presence and absence of light. From these data, authors concluded that no differences in terms of acute toxicity existed between irradiated and non irradiated exposures. In a study by Ohji & Harino (2017) individuals of the fish species *Oryzias latipes* were exposed to two concentrations, 1 and 10 μg/l, of copper pyrithione, zinc pyrithione, and to their degradation products. Toxicity was evidenced in the first 12 hours, with 100% mortality for copper pyrithione in animals exposed to the two concentrations (1 and 10μg/l) and to the highest concentration (10μg/l) of zinc pyrithione; however, for the degradation products, the concentrations analyzed did not cause mortality for 48 hours, but changed
respiration pattern and caused swimming abnormalities. This study demonstrated the higher toxicity of copper pyrithione vs. zinc pyrithione, while acute effects of the degradation products of the two pyrithiones were less pronounced than those caused by the parent compounds. The highest toxicity of copper pyrithione over zinc pyrithione was also reported by Mochida et al. (2006) with a marine fish species (*Pagrus major*) and a crustacean (*Heptacarpus futilirostris*). The evaluation of toxicity of copper and zinc pyrithione mixtures was performed with *Artemia salina* as test organism. The EC$_{50}$ was calculated for the two compounds with values of 830 μg/l for zinc pyrithione and 1000 μg/l for copper pyrithione and synergistic effects were observed when organisms were exposed to the mixture of both pyrithiones (Koutsaftis & Aoyama, 2007). Synergistic effects were also observed after the exposure of diatoms, *Thalassiosira pseudonana*, polychaete larvae, *Hydroids elegans* and amphipods *Elasmopus rapax* to combinations of zinc and copper pyrithione (Bao et al., 2008). However, even considering the possibility of translocation of zinc pyrithione into its copper form, it can not be assumed that, under the conditions adopted in the present study, this hypothesis could be responsible for considerable increase in the toxicity of zinc pyrithione. From the here mentioned studies, it becomes clear that distinct organism present different sensitivity toward zinc pyrithione, and that low levels are required for the exertion of acute effects in some cases. However, for the here studied species, the levels of zinc pyrithione that are required to cause acute toxic effects, such as mortality, exceed by far those found at real scenarios of contamination.

Exposure to zinc pyrithione appears to be associated with increased oxidative stress. The ability of zinc pyrithione to cause oxidative damage was studied using rat thymocytes. Zinc pyrithione has been shown to increase lethality in cells that undergo induced oxidative stress. Zinc pyrithione alone increased the level of intracellular zinc, the amount of superoxide anions, and the simultaneous application of hydrogen peroxide resulted in synergistic effects, also indicating a severe cytotoxic action (Oyama et al., 2012). In the present study, zinc pyrithione triggered antioxidant defenses in *D. magna* by altering the enzymatic activities of GSTs and catalase, and in *D. longispina* by altering the activity of GSTs alone. Whereas exposure to zinc pyrithione has taken place at environmentally relevant concentrations (5 to 19,2 μg/l), findings of this nature are important to understand the environmental effects caused by that substance. In *D. magna*, increased catalase and GSTs indicate the potential of zinc pyrithione of causing oxidative alterations. The
activation of both defense mechanisms indicates the overproduction of reactive oxygen species, leading to a scenario of imbalance between the production and scavenging of ROS, which can trigger adaptive short- and long-term individual effects. This is extremely relevant if one takes into account that the concentrations used in our experimental design can be found in the aquatic environment. In D. longispina, zinc pyrithione did not trigger alterations in catalase activity, suggesting that there was not sufficient hydrogen peroxide production to alter this enzyme activity; further inhibition of GSTs was observed. However, zinc pyrithione metabolism may end up in oxidative modifications in D. magna, including enzymatic inhibition. In addition, the inhibition of a phase II enzyme is of concern, since it is one of the xenobiotic detoxification pathways for most xenobiotics.

The few existing studies that focused on the neurotoxicity of zinc pyrithione used fish as a model organism; consequently extrapolations from such results to the here-adopted approach are somewhat difficult, since the level of complexity and organization of nervous networks of fish do not find a suitable and comparable counterpart in microcrustacean species such as Daphnia. Fish of the species Fundulus heteroclitus were exposed to zinc pyrithione and to its degradation products. Exposed organisms evidenced inhibition of acetylcholinesterase only after exposure to its degradation products, without changes caused by the parent compound (Mochida et al., 2009). These results were consistent with the study carried out in Gambusia holbrooki exposed to zinc pyrithione, where no changes were observed in acetylcholinesterase activity (Nunes et al., 2015). However, these tests were performed in the absence of light, not allowing its photodegradation and the generation of potentially neurotoxic photodegradation products. In the present work, zinc pyrithione inhibited acetylcholinesterase in the two species studied, an effect that was however more pronounced in the case of D. magna. The experimental exposures were performed in the presence of light, and theoretically degradation products may have been formed, which may justify the results obtained. However, neutotoxic effects may have not resulted from such presence, since an alternative inhibitory pathway, already described, may have also have affected cholinesterasic activity of exposed organisms. In fact, and considering that zinc pyrithione showed a great capacity to cause the activation of antioxidant defenses in D. magna, and enzymatic alterations in D. longispina that may be associated with the production of reactive oxygen species, our results suggest that the neurotoxic effects of zinc pyrithione can be a conjugation of direct inhibitory effects by
photodegradation products of zinc pyrithione, and direct effects of reactive species on enzymes, causing their inhibition as discussed in the previous section (Delwing-de Lima et al., 2010; Weiner et al., 1994). Exposure of *D. longispina* to ZnPT resulted in a significant inhibitory effect, but only for an intermediate concentration, suggesting that, despite potentially evidencing neurotoxic effects, *D. longispina* was less responsive than *D. magna* towards this chemical. However, and despite the ecological relevance of the here obtained results (these modifications occurred for low levels of zinc pyrithione), data in the literature are scarce, and additional studies with other species are necessary to deepen the knowledge about neurotoxic effects of zinc pyrithione under realistic settings, of exposure concentrations and abiotic conditions (e.g. light).

Locomotion is a feature usually compromised by a series of toxicants but seems to be rather unspecific. In fact, changes in mobility of living organisms have been associated to various environmental factors, such as light, water temperature, food presence and predators (Hamza & Ruggiu, 2000; Ziarek et al., 2011), but may be also changed by exposure to pesticides (Dodson et al., 1995; Duquesne & Küster, 2010), hormonal drugs (Goto & Hiromi, 2003), cyanobacterial toxins (Ferrão-Filho et al., 2014; Restani & Fonseca, 2014), metals such as cadmium (Wolf et al., 1998), among others. In the present study, swimming traits of exposed organisms were not altered following a clear dose-response pattern, and the changes were diffuse in relation to the exposure parameters and cycles, with some moments of excitation (increase in distance and time) or inhibition (decrease in distance and time). These findings are somewhat paradoxical considering that cholinesterasic activity seemed to be considerably impacted after exposure to zinc pyrithione, and in both species. Cholinesterase plays an important role in the functioning of the cladoceran neuronal system (Solé et al., 2010). Studies with *D. magna* indicated the relationship between inhibition of cholinesterases activity and behavioral changes. Duquesne & Küster (2010) established this relationship after exposing individuals of this species to organophosphorus insecticides. Cladocerans have a neural system or primitive neurological mechanisms, and their cholinesterases seems to respond to a diversity of xenobiotics, including drugs (Solé et al., 2010). The changes in cholinesterase activity were observed in neonates and in this study, only adults of *D. magna* showed behavioral changes, so these results may be associated with other stress factors. Consequently, we cannot conclude that behavioral changes that were ascertained in *D. magna* were directly
related to the potential neurotoxic effects of zinc pyrithione, since environmental effects (such as light) seemed also to exert a decisive role in behavioral activity of organisms of this species. In fact, no concrete dose-response relationships were detected concerning behavioral alterations or cholinesterasic impairments, suggesting that no connection between these two parameters was ever clearly established. Indeed, neurotoxic potential of zinc pyrithione can not be ruled out – especially in *D. longispina*, behavioral changes were observed at both ages. In this species, behavioral changes seemed to be directly related to the neurotoxic potential of zinc pyrithione, due to the alteration of acetylcholinesterase activity probably due to the exposure of anticholinergic phetodegradation products of zinc pyrithione. In addition, and as already suggested, inhibition of cholinesterase activity may also be associated with direct reaction of reactive species with enzymes (Weiner et al., 1994; Delwing-de Lima et al., 2010;). However, in this species, the antioxidant defenses did not appear to have been fully activated after exposure to zinc pyrithione, as there were no changes in catalase activity. This does not exclude the occurrence of pro-oxidant alterations of another type, since enzymatic inhibition may result from reactive species acting on enzymes and causing their denaturation. In fact, and for this particular species, GSTs and cholinesterases were inhibited. Despite the scarce literature about this issue, and the now generated limited amount of data, the here obtained prospective results reinforce the notion about the sensitivity of these two organisms, which after being exposed to sublethal concentrations of zinc-pyridithione, may suffer effects in terms of their main physiological functions. These results are relevant because changes in locomotor activity may interfere with key behavioral features (escape, camouflage, vertical migration), thereby critically disturbing the predator-prey relationship, causing changes in the population dynamics of both species (Dodson et al., 1995).

Effects on population dynamics such as decreased age and size of primiparous animals, changes in litter number and size are reproductive defenses induced in *Daphnia* by stressors such as the presence of predators (De Meester & Weider, 1999) and xenobiotics (Burks et al., 2002), with costs to the individual at the metabolic, physiological or reproductive level (Lass & Spaak, 2003). Population effects are thus unspecific alterations, caused by a diverse array of factors, but it is well known from the literature that several xenobiotics modulate the reproductive response in several species of the genus *Daphnia.* Effects on fertility reduction were observed in *D. longispina* after exposure to the herbicide
Primextra® Gold TZ (Neves et al., 2015) and zinc (Martins et al., 2017) and in *Daphnia magna* after exposure to a cocaine metabolite (benzoylecgonine) (Parolini et al., 2018), bisphenol A (Masteling et al., 2016), hormonal drugs (Goto & Hiromi, 2003) and non-steroidal anti-inflammatory drugs (paracetamol, ibuprofen and diclofenac) (Du et al., 2016). Although several xenobiotics modulate reproductive response in the genus *Daphnia*, in this study zinc pyrithione was not able to induce any measurable alteration. However, behavioral alterations and alterations at the biochemical level were evidenced, suggesting that the adopted defensive mechanisms may have prevented the onset of population effects. This is obviously of undisputable ecological relevance since it suggests that the actual levels of this drug in the wild may not attain levels required to result in populational challenges.

In addition, it must also be emphasized that the two here tested species of cladocerans exhibited dissimilar responses to the same chemical, ZnPT. In fact, and considering the obtained results for mortality and also for the physiological/biochemical parameters, *D. magna* was always more sensitive than *D. longispina*, evidencing critical species-specific responses that strongly modulated the toxic response and its patterns. This is a significant finding, since more sensitive species are naturally more likely to be impacted by the presence of xenobiotics in the wild. However, this comparison of sensitivity between *D. magna* and *D. longispina* has not always showed that the first species is more sensitive. In fact, the work by Gonçalves et al. (2007), evidenced that *D. magna* was less responsive to salinity than *D. longispina*, but only when reproductive traits were analysed. In a similar study, Antunes et al. (2004) showed that *D. longispina* individuals were again more sensitive to the pesticide lindane when compared to *D. magna*, after evaluating their life history traits. Even exposures to pharmaceutical drugs have resulted in a similar pattern of toxicity, *D. longispina* being always more sensitive than *D. magna*. The exposure of *D. longispina* to acetylsalicylic acid resulted in more pronounced effects, when compared to *D. magna*, on life history traits, as evidenced by Marques et al. (2004a,b). Despite being inherently distinct (our data focused on physiological adaptive mechanisms, and these findings from the literature focused on reproduction and life history traits), it is possible to anticipate that sensitivity of cladocerans depends mostly on the studied stressor, rather than being a generic rule that implies that one species is always more sensitive than other. On the other hand, the provenance of organisms is a key aspect when comparing sensitivity.
among species and clones. *D. magna* is a standard test organism, which has been cultured for decades under optimal laboratory conditions to serve as test organisms in a multiplicity of assays, but even such conditions may have a decisive effect on the quality of results (Baird et al., 1989). *D. longispina* is an autochtonous species that, despite being kept under strictly controlled conditions in the laboratory, may be strongly influenced by its origins. Clones of *D. longispina* isolated from field populations (such as the one used in the here-described bioassays), are likely to have strong variations in their physiological responses towards chemicals or other abiotic/environmental stressors. The study by Antunes et al. (2003) evidenced that food levels could play a decisive role in the fitness and toxic responses of distinct clones of *D. longispina*. In addition, genetic erosion of wild *D. longispina* populations caused by environmental exposure to chemical contaminations could strongly modulate the efficacy and robustness of the toxic response towards other stressors, alone or in combination (such as copper and salinity, as shown by Leitão et al., 2013). Consequently, clones of *D. longispina* coming from different geographical locations, and probably previously exposed to distinct sets of contaminants, may have dissimilar responses to toxicants.

**Conclusions**

Pharmaceutical compounds may exert subtle chronic effects which may occur at the biochemical level, which may extend to populations and communities, not triggering visible lethal acute effects but activating imperceptible toxicity mechanisms. This study attempted to address the different levels susceptible to produce effects triggered by zinc pyrithione, evaluating mortality, swimming behavior and biochemical parameters (antioxidant defenses, metabolism, and neurotoxicity) and reproduction in two species of cladocerans, *D. magna* and *D. longispina*. Interspecific differences were observed in our results with greater sensitivity being attributed to *D. magna* in relation to the observed toxicity. Zinc pyrithione was able to trigger oxidative and neurotoxic effects in the species studied. However, the relationship between behavioral changes in *D. magna* and neurotoxicity are not conclusive, since the enzymatic determinations were performed in neonates, and for this species no significant changes were observed at this age. The results demonstrate that zinc pyrithione is a potential threat to aquatic organisms in relation to
zooplanktonic species, such as cladocerans. Results of this nature and magnitude are cause of concern, since acute exposures in environmentally relevant concentrations have triggered biochemical and functional changes, indicating that significant toxic effects can be induced by these drugs at the environmental level.

Credit Author Statement

Ana Paula Sousa was involved in the following tasks which lead to the writing of this article: conceptualization; Investigation; Methodology; Validation; Visualization; Writing - original draft;

Bruno Nunes had the following responsibilities: conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions

Fig. 1. Catalase activity (CAT) measured in *D. magna* (light bars) and *D. longispina* (dark bars) after acute exposure (48h) to zinc pyrithione, n = 5, error bars correspond to standard error and * represents statistically significant differences (Dunnett’s test, p<0.05) between the different concentrations and the negative control.

Fig. 2. Activity of glutathione S-transferases (GSTs) quantified in *D. magna* (light bars) and *D. longispina* (dark bars) after acute exposure (48h) to zinc pyrithione, n=5, error bars correspond to standard error and * represents statistically significant differences (Dunnett’s test, p<0.05) between the different concentrations and the negative control.

Fig. 3. Activity of cholinesterases (ChEs) measured in *D. magna* (light bars) and *D. longispina* (dark bars) after acute exposure (48h) to zinc pyrithione, n=5, error bars correspond to standard error and * represents statistically significant differences (Dunnett test, p<0.05) between the different concentrations of zinc pyrithione and the negative control.

Fig. 4. Behavior observed in *D. magna* (24h and 6 days) after exposure to zinc pyrithione, n=20. Left - average swimming time (ST) and average swimming distance (SD) covered. Right - total swimming time average and distance covered average, error bars correspond to standard error and * represents statistically significant differences (Dunnett test, p <0.05) between the different concentrations of zinc pyrithione and the negative control.

Fig. 5. Behavior observed in *D. longispina* (24h and 6 days) after exposure to zinc pyrithione, n=20. Left - average swimming time (ST) and average swimming distance (SD) covered. Right - average swimming time (ST) and average swimming distance (SD) covered, error bars correspond to standard error and * represents statistically significant differences (Dunnett test, p <0.05) between the different concentrations of zinc pyrithione and the negative control.

Fig. 6. Average number of neonates released in the reproduction test for the two species of *Daphnia*. 
Figure 1

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Table legends

Table 1. 48h EC$_{50}$ values, and respective confidence intervals (CI) of 95%.

<table>
<thead>
<tr>
<th>Paracetamol (mg/l)</th>
<th>Species</th>
<th>EC$_{50}$ (48h)</th>
<th>lowest</th>
<th>highest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D. magna</td>
<td>3.18</td>
<td>2.94</td>
<td>3.46</td>
</tr>
<tr>
<td></td>
<td>D. longispina</td>
<td>30.45</td>
<td>26.12</td>
<td>37.05</td>
</tr>
</tbody>
</table>
Table 2. Statistical analysis of the behavioral test after exposure to zinc pyrithione, where TT - total swimming time (s), TD - total distance traveled (mm), 300 and 900 (light cycle), 600 and 1200 (dark cycle), with n = 20 and degrees of freedom (5; 114). Values of F and p are inserted according to the cycles, age of the organisms and compound analyzed.

<table>
<thead>
<tr>
<th>Paracetamol</th>
<th>CYCLES</th>
<th>D. magna (24h)</th>
<th></th>
<th>D. magna (6 days)</th>
<th></th>
<th>D. longispina (24h)</th>
<th></th>
<th>D. longispina (6 days)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>p</td>
<td></td>
<td>F</td>
<td>p</td>
<td></td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>TT-300</td>
<td>3,958</td>
<td><strong>0.002</strong></td>
<td></td>
<td>3,191</td>
<td><strong>0.01</strong></td>
<td></td>
<td>2,296</td>
<td>0.052</td>
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<tr>
<td>TT-600</td>
<td>4,521</td>
<td><strong>0.001</strong></td>
<td></td>
<td>7,213</td>
<td><strong>0.001</strong></td>
<td></td>
<td>1,292</td>
<td>0.272</td>
<td></td>
</tr>
<tr>
<td>TT-900</td>
<td>2,633</td>
<td><strong>0.027</strong></td>
<td></td>
<td>2,015</td>
<td>0.082</td>
<td></td>
<td>5,046</td>
<td><strong>0.05</strong></td>
<td></td>
</tr>
<tr>
<td>TT-1200</td>
<td>2,900</td>
<td><strong>0.017</strong></td>
<td></td>
<td>5,254</td>
<td><strong>0.001</strong></td>
<td></td>
<td>0.881</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td>TD-300</td>
<td>1,319</td>
<td><strong>0.03</strong></td>
<td></td>
<td>3,044</td>
<td>0.08</td>
<td></td>
<td>4,116</td>
<td><strong>0.002</strong></td>
<td></td>
</tr>
<tr>
<td>TD-600</td>
<td>0.924</td>
<td>0.468</td>
<td></td>
<td>12.94</td>
<td><strong>0.001</strong></td>
<td></td>
<td>3.84</td>
<td><strong>0.003</strong></td>
<td></td>
</tr>
<tr>
<td>TD-900</td>
<td>1,112</td>
<td>0.358</td>
<td></td>
<td>2,953</td>
<td><strong>0.015</strong></td>
<td></td>
<td>10,134</td>
<td><strong>0.001</strong></td>
<td></td>
</tr>
<tr>
<td>TD-1200</td>
<td>1,318</td>
<td>0.262</td>
<td></td>
<td>10,986</td>
<td><strong>0.001</strong></td>
<td></td>
<td>4,805</td>
<td><strong>0.001</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Behavioral changes observed in *D. magna* and *D. longispina* after exposure to zinc pyrithione, TT - total swimming time (s), TD - total distance traveled (mm), 300 and 900 (light cycle), 600 and 1200 (dark cycle), (↑) - increase in relation to the control, (↓) - decrease in relation to the control, and * represents statistically significant differences (Dunnett test, p < 0.05) between the different concentrations of acetaminophen and zinc pyrithione in relation to the negative control.

| Paracetamol | 5  | 10 | 20 | 40 | 80 | 5  | 10 | 20 | 40 | 80 |
|-------------|----|----|----|----|----|----|----|----|----|----|----|
| Concentration (µg/l) | Daphnia magna | Daphnia longispina |
| TT-300 | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* |
| TT-900 | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* |
| TT-600 | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* |
| TT-1200 | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* |
| TD-300 | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* | *(↑)* |
| TD-900 | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* |
| TD-600 | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* |
| TD-1200 | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* | *(↓)* |